IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Akihito TSUCHIYA.

Appln. No. 09/731,863 Group Art Unit: 1744

Filed: 12/08/2000 Examiner: BEISENER, WILLIAM H

For: METHOD FOR PURIFICATION TREATMENT OF ENVIRONMENT POLLUTANT

10
3/27/03
ds

DECLARATION

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir :

I, Akihito TSUCHIYA, hereby declare that:

1) I currently reside at 116-1, Chuo 5-chome, Kosei-cho, Koga-gun, Shiga-ken, Japan. I am currently employed by ALLMIGHTY CO., LTD

2) I am a inventor of the instant invention, and

3) The experiments given below were carried out under my general direction and supervision.

Experiment

1. Purpose of the experiment

This experiment aims to examine, in the purification of an environmental pollutant using a microbial treatment agent, how the type of organic polymer used to incorporate microorganisms influences the degradation rate of the environmental pollutant.

2. Method of the experiment

2.1 Preparation of microorganisms

A liquid medium (composition: 0.5 wt.% of yeast extract, 1.0 wt.% of bacttryptone and 1.0 wt.% of sodium chloride) (150 L) was placed in a 300-liter fermenter (MPF-U type 300-liter fermenter; B. E. Marubishi Co., Ltd.), steam-sterilized (121°C, 20 min), and adjusted to 30°C. Then, *Bacillus subtilis* isolated from sewage sludge was inoculated into the medium and incubated for 48 hours under aerobic conditions with agitation at 200 rpm.

The culture solution thus obtained was centrifuged at 3,000 rpm to collect the bacterial cells, which were then dried to obtain a dry bacterial cell powder.

2.2 Preparation of microbial treatment agents

<Alginic acid-containing microbial treatment agent>

Sodium alginate (Kishida Chemical Co., Ltd.) (0.1 g) was added to 5 mL of deionized water heated to 98°C, and the resulting mixture was allowed to stand at room temperature to fully swell the alginic acid. When the temperature of the alginic acid-containing solution was lowered to 80°C, the solution was gently stirred to dissolve the alginic acid. Separately, using the bacterial cells obtained above, a bacterial cell solution with a concentration of 0.25 g (dry bacterial cell weight)/ml was prepared. The bacterial cell solution (4 mL) was added to the alginic acid-containing solution, followed by stirring to homogeneity. The bacterial cell/alginic acid suspension thus obtained was added dropwise to a 0.1M $CaCl_2$ solution to form a precipitate, which was collected to obtain an

alginic acid-containing microbial treatment agent. The microbial treatment agent contained 1.01 x 10^8 cells per gram.

<Carrageenan-containing microbial treatment agent>

A carrageenan-containing microbial treatment agent was prepared by following the procedure for preparing the alginic acid-containing microbial treatment agent, except using carrageenan (Kishida Chemical Co., Ltd.) in place of alginic acid. The obtained microbial treatment agent contained 1.13 x 10^8 cells per gram.

<Chitosan-containing microbial treatment agent>

Chitosan (Chitosan S, Taiyo Chemical Industrial Co., Ltd.) (1.5 g) was added to 10 mL of deionized water. While stirring the resulting mixture, a 0.1N H_2SO_4 solution was further added to adjust the pH to 5.0. The resulting chitosan dispersion was filtered through Toyo filter paper No. 5 (Toyo Filter Paper Co.) to collect the solids. The solids were added to 50 mL of deionized water and fully dispersed to prepare a chitosan-containing solution. Separately, using the bacterial cells obtained above, a bacterial cell solution with a concentration of 0.8 mg (dry bacterial cell weight)/ml was prepared. The bacterial cell solution (1.5 mL) was added to the chitosan-containing solution, followed by stirring to homogeneity. The bacterial cell/chitosan suspension thus obtained was adjusted to pH 5 with 1N H_2SO_4, filtered through Toyo filter paper No. 5 (Toyo Filter Paper Co.), giving a chitosan-containing microbial treatment agent. The

microbial treatment agent contained 1.20×10^8 cells per gram.

<Polyamino acid-containing microbial treatment agent>

Polyamino acid (L-glutamic acid-γ-benzyl ester having a molecular weight of 220,000; CPR Co., Ltd.) (5.5 g), acetic acid (0.03 g), and Triton X-100 (Wako Pure Chemical Industries, Ltd.) (0.2 g) were added to 10 mL of deionized water heated to 20°C to prepare a polyamino acid solution. The bacterial cells prepared above (1.2 mg) were added to the polyamino acid solution, followed by stirring at 37°C for 25 minutes. Then, acetone (4 mL) was added to form a precipitate, which was collected by centrifugation to obtain a polyamino acid-containing microbial treatment agent. The microbial treatment agent contained 1.10×10^8 cells per gram.

<*Bacillus* bacteria-produced mucin-containing microbial treatment agent>

Bacillus natto isolated from commercially available fermented soybeans was inoculated into 2.5 L of a liquid medium (containing 5 wt.% of glucose, 8 wt.% of sodium glutamate, 0.4 wt.% of peptone, and 0.2 wt.% of K_2HPO_4), and aerobically incubated at 30°C for 5 days. The bacterial cells were removed from the culture solution by centrifugation, and an aqueous methanol solution (containing 80 wt.% of methanol) (3.5 L) was added to the obtained culture supernatant to form a precipitate. The precipitate was collected by centrifugation, washed with

methanol, and vacuum-dried, giving a *Bacillus* bacteria-produced mucin powder.

The *Bacillus* bacteria-produced mucin powder (0.1 g) was added to 5 mL of deionized water heated to 20°C to fully swell the powder. To the resulting *Bacillus* bacteria-produced mucin solution, 1.2 mg of the bacterial cells obtained above was added, followed by stirring to homogeneity. The bacterial cell/*Bacillus* bacteria-produced mucin suspension thus obtained was added dropwise to a 0.1M $CaCl_2$ solution to form a precipitate, which was collected to obtain a *Bacillus* bacteria-produced mucin-containing microbial treatment agent. The microbial treatment agent contained 1.16×10^8 cells per gram.

<*Zoogloea* bacteria-produced polysaccharide-containing microbial treatment agent>

The microorganism of the genus *Zoogloea* isolated from a sewage treatment plant by the method of Mckinney et al. (Mckinney, R. E. and Weichlein, R. G.: Applied Microbiology, vol. 1, p. 259, 1953) was inoculated into 20 mL of synthetic sewage described below, and aerobically incubated at 30°C for 30 days. The bacterial cells were removed from the culture solution by centrifugation. To the obtained culture supernatant, the same amount of ethanol as the supernatant was added to form a precipitate. The precipitate was collected by centrifugation, washed with an aqueous ethanol solution (containing 50 wt.% of ethanol), and vacuum-dried, giving a *Zoogloea* bacteria-produced polysaccharide.

Then, 0.1 g of the *Zoogloea* bacteria-produced polysaccharide was added to 5 mL of deionized water heated to 20°C to fully swell the polysaccharide. To the resulting polysaccharide solution, 1.2 mg of the bacterial cells obtained above was added, followed by stirring to homogeneity. The bacterial cell/*Zoogloea* bacteria-produced polysaccharide suspension thus prepared was added dropwise to a 0.1M $CaCl_2$ solution to form a precipitate, which was collected to obtain a *Zoogloea* bacteria-produced polysaccharide-containing microbial treatment agent. The microbial treatment agent contained 1.05×10^8 cells per gram.

2.3 Preparation of synthetic sewage

4-Octylphenol was added as an environmental pollutant, at a concentration of 150 ppm, to an aqueous solution of the following composition (COD: 102 ppm, total nitrogen content: 32 ppm, total phosphorus content: 3.5 ppm) to prepare synthetic sewage.

Glucose	0.5	g
K_2HPO_4	0.004342	g
KH_2PO_4	0.0017	g
$Na_2HPO_4 \cdot 12H_2O$	0.00892	g
NH_4Cl	0.04674	g
$MgSO_4 \cdot 7H_2O$	0.0225	g
$FeCl \cdot 6H_2O$	0.00025	g
$CaCl_2$	0.0275	g
Polypeptone	0.15	g
Deionized water	Balance	
Total	1 L (pH 7.0)	

2.4 Degradation test of the environmental pollutant

Each of the microbial treatment agents prepared above was added, at a concentration of 500 ppm, to 20 mL of the synthetic sewage, and incubated at 30°C for 1 week with shaking. After the incubation, 10mL of supernatant was drawn from the synthetic sewage after the degradation treatment. To the remainder of the synthetic sewage after the degradation treatment, 10 mL of untreated synthetic sewage was added, and incubation was carried out under the same condition as above. Such operation was continued for 5 weeks (total 5 times).

3. Result of the experiment

Table 1 shows the remaining proportion (%) of 4-octylphenol after the treatment with each microbial treatment agent, relative to the initial 4-octylphenol concentration.

	4-Octylphenol remaining proportion (%)				
	After 1 week	After 2 weeks	After 3 weeks	After 4 weeks	After 5 weeks
Polyamino acid	99.0	98.1	97.4	78.2	80.4
Bacillus bacteria-produced mucin	98.1	99.0	96.8	90.1	76.2
Zoogloea bacteria-produced polysaccharide	99.2	98.8	98.2	92.2	80.6
Alginic acid	99.8	101.1	100.8	99.9	100.2
Carrageenan	99.8	100.5	101.2	104.1	100.0
Chitosan	99.9	102.0	101.0	98.1	102.0

4. Consideration

The results shown in Table 1 confirm that a microbial treatment agent prepared using the specific microorganism-produced polymer (polyamino acid, *Bacillus* bacteria-produced mucin, or *Zoogloea* bacteria-produced polysaccharide) can degrade 4-octylphenol more effectively than that prepared using alginic acid, carrageenan, or chitosan. This matter is clearly demonstrated by the 4-octylphenol remaining proportion after 5 weeks. Thus, it is presumed that, owing to the effects of the specific microorganism-produced polymer, the microorganisms retain their biological activity necessary for degrading 4-octylphenol.

The test results reveal that the use of the specific microorganism-produced polymers for incorporating microorganisms enables more effective degradation of an environmental pollutant.

I, the undersigned, declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: March 3, 2003 /Chris Tucker/